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NOTES

Functional Analysis of DNA Gyrase Mutant Enzymes Carrying Mutations at Position 88 in the A Subunit Found in Clinical Strains of *Mycobacterium tuberculosis* Resistant to Fluoroquinolones[▽]

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We investigated the enzymatic efficiency and inhibition by quinolones of *Mycobacterium tuberculosis* DNA gyrases carrying the previously described GyrA G88C mutation and the novel GyrA G88A mutation harbored by two multidrug-resistant clinical strains and reproduced by site-directed mutagenesis. Fluoroquinolone MICs and 50% inhibitory concentrations for both mutants were 2- to 43-fold higher than for the wild type, demonstrating that these mutations confer fluoroquinolone resistance in *M. tuberculosis*.

The incidence of multidrug-resistant tuberculosis (MDR-TB), which is associated with high rates of mortality (7), has become a concern for TB control in many countries (23). Fluoroquinolones are one of the three bactericidal drug groups recommended for the treatment of MDR-TB (3, 11, 23). Unfortunately, acquired resistance to quinolones in *Mycobacterium tuberculosis* (5, 8, 18, 22), described since the first use (6), is increasing (12).

DNA gyrase, a tetrameric A_2B_2 protein acting by a transient double-stranded DNA break and cooperating to facilitate DNA replication and other key DNA transactions, is the sole target for quinolones in *M. tuberculosis* (9, 10, 14). Mutations involved in *M. tuberculosis* quinolone resistance described so far generated substitutions in the A subunit at positions 90, 91, and 94 and more rarely in the B subunit at position 510, as described for other bacteria (2, 6, 8, 18, 20, 22). These mutations confer cross-resistance to all members of the quinolone family (20).

Because fluoroquinolone therapy is associated with an improved outcome in MDR-TB (7, 23), each MDR *M. tuberculosis* clinical strain submitted to our laboratory is tested for fluoroquinolone susceptibility. In this context, we identified a new mutation leading to G88A in GyrA in two MDR-*M. tuberculosis* strains isolated from patients with pulmonary tuberculosis who relapsed after being treated by a fluoroquinolone, either ofloxacin or moxifloxacin. Even for bacteria other than *M. tuberculosis* (4, 15, 19, 20), the effect of mutations at position 88 in GyrA on quinolone susceptibility is poorly understood, and no study clearly demonstrated the implication of these mutations in quinolone resistance. We aimed to examine the effects at the molecular level of amino acid substitutions at position 88 in GyrA by expressing recombinant DNA gyrases harboring mutations at this position. These mutations lead to fluoroquinolone resistance in *M. tuberculosis*, but the resistance level depends on the quinolone structure with regard to the substitutions at the residues R6 and R7. Our observations bring new insight to the role of position 88 in the model of quinolone binding.

MICs were determined by the 1% standard proportion method on 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase for the two *M. tuberculosis* clinical isolates bearing GyrA G88A mutations and the quinolone-susceptible strain *M. tuberculosis* H37Rv (13).

Plasmids expressing *M. tuberculosis gyrA* genes containing the new mutation G88A or the mutation G88C, previously described (17, 18, 22), were generated from pATB (1), which contained the wild-type (WT) *gyrA* gene of *M. tuberculosis*, by using the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Oligonucleotides synthesized by MWG-Biotech (Ebersberg, Germany) mimicked nucleotides 247 to 282 of the *M. tuberculosis gyrA* sequence containing the appropriate base substitutions (in boldface characters): G88A (5′ AACTACCACCCGCACG**C**C GACGCGTCGATCTACGAC 3′) and G88C (5′ AACTACCA CCCGCAC**T**GCGACGCGTCGATCTACGAC 3′), together with complementary strands 5′ GTCGTAGATCGACGCGTCG **G**CGTGCGGGTGGTAGTT 3′ for G88A and 5′ GTCGTAGA TCGACGCGTCGC**A**GTGCGGGTGGTAGTT 3′ for G88C.

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TABLE 1. Quinolone and coumarin activities against *M. tuberculosis* WT and mutated strains (MICs) and DNA gyrases (IC_{50}s)

Drug	MIC (μg/ml)		IC_{50} (μg/ml)			
	WT	G88A[a]	WT	G88A	G88C	D94H
Quinolones						
Sparfloxacin	0.25	0.5; 1	2.5	10	32	ND
Gemifloxacin	4	32; 64	3.5	90	150	ND
Gatifloxacin	0.12	1	4	7	>128	150[b]
Moxifloxacin	0.25	2	4	10	35	90[b]
Levofloxacin	0.5	1	5	30	100	320[b]
Ofloxacin	0.5	2	10	40	50	800[b]
Temafloxacin	4	8	30	70	250	ND
Enoxacin	4	64	100	320	320	ND
Flumequine	64	32; 128	700	650	600	>2,000
Oxolinic acid	32	16; 32	800	800	800	ND
Nalidixic acid	128	64; 128	1,100	1,200	1,150	>3,200
Pipemidic acid	256	256	2,200	2,000	1,500	ND
Coumarin						
Novobiocin	ND[c]	ND	0.3	0.3	0.2	ND

[a] When the MICs were different for the two clinical strains, both values are presented.
[b] From Aubry et al. (2).
[c] ND, not determined.

WT and mutant GyrA subunits were purified as previously described, as was the WT GyrB subunit. DNA supercoiling experiments were carried out as described previously (1, 2).

The two *M. tuberculosis* clinical strains carrying the G88A mutation were resistant to fluoroquinolones, the MICs of which were 2- to 16-fold higher than those for the WT H37Rv reference strain (Table 1). These two strains harbored a level of fluoroquinolone resistance lower than those of strains described with the GyrA G88C mutation, i.e., a laboratory-selected mutant of *M. tuberculosis* H37Rv for which the ciprofloxacin MIC was over 3 μg/ml (22) and a clinical strain isolated from a patient treated with ofloxacin as a monotherapy for which the levofloxacin MIC was 16 μg/ml (17).

The recombinant gyrase complexes bearing GyrA G88A and G88C were 2- to 26- and 3- to 43-fold more resistant than WT gyrase to inhibition by fluoroquinolones, respectively (Table 1; Fig. 1). The GyrA G88C mutant showed fluoroquinolone 50% inhibitory concentrations (IC_{50}s) similar to that observed for the classical GyrA A90V mutant well known to be implicated in fluoroquinolone resistance in *M. tuberculosis* (2). These results establish clearly that the GyrA G88C and G88A mutations confer resistance to fluoroquinolones in *M. tuberculosis* by decreasing gyrase inhibition. However, even if both mutations are implicated in fluoroquinolone resistance, increases in IC_{50}s were higher for the G88C mutant than for the G88A mutant for sparfloxacin, gemifloxacin, gatifloxacin, moxifloxacin, levofloxacin, and temafloxacin and were similar for ofloxacin and enoxacin (Table 1).

Glycine at position 88 in *M. tuberculosis*, which corresponds to the glycine at position 81 in *Escherichia coli*, is a small and flexible amino acid lying at the N-terminal end of the α4 helix, which may be part of the drug binding site (16). Since alanine is a hydrophobic amino acid whereas cysteine is polar and more bulky than alanine and glycine, it is consistent that the level of resistance to fluoroquinolone inhibition observed with the gyrase harboring GyrA G88C was higher than with the gyrase harboring GyrA G88A.

Intriguingly, for the four classical quinolones tested which do not have either an R7 ring (nalidixic acid and flumequine) or a fluor in R6 (oxolinic acid, pipemidic acid, and nalidixic acid), MICs were similar for the G88A mutants and the H37Rv strain, and IC_{50}s of both G88C and G88A mutant gyrases were similar to the IC_{50}s of the WT gyrase (Table 1; Fig. 1). These results are consistent with the observation of the mutation G81D in *E. coli* that conferred resistance to ciprofloxacin but susceptibility to nalidixic acid (4), whereas mutations at positions 83, 84, and 87 in *E. coli* conferred resistance to ciprofloxacin as well as to nalidixic acid (20). Since resistance to quinolones is usually crossed between all drugs of the family (20), we controlled these results by measuring IC_{50}s of these four quinolones for the mutant gyrase with a GyrA D94G known to be implicated in fluoroquinolone resistance in *M. tuberculosis* (2). For the D94G mutant, IC_{50}s of flumequine and nalidixic acid were at least threefold higher than for the WT and reached >2,000 and >3,200 μg/ml, respectively (Table 1).

In *E. coli*, G81 is assumed to interact with the R7 fluoro-

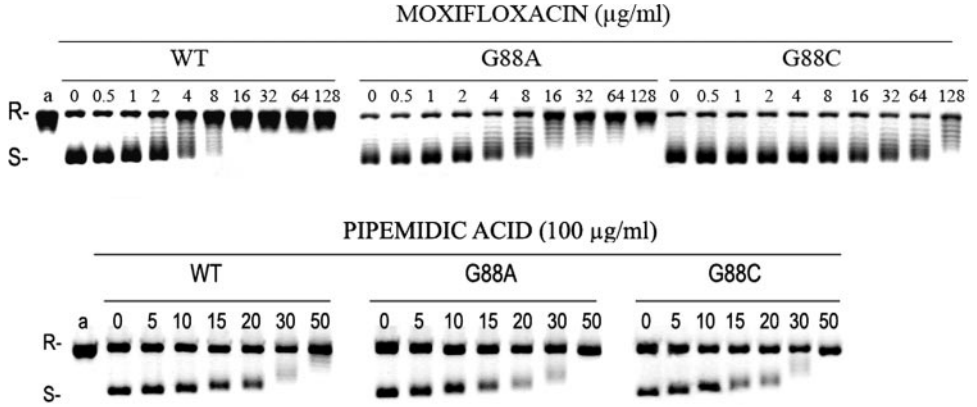

FIG. 1. Inhibitory activity of moxifloxacin and pipemidic acid on the supercoiling activity of *M. tuberculosis* DNA gyrase and wild-type (WT) and mutant proteins carrying GyrA mutations at position 88. Relaxed pBR322 DNA (0.4 μg) was incubated with gyrase (2 U) reconstituted from WT GyrA with WT GyrB and from WT GyrB with G88A or G88C GyrA. Incubation was carried out in the presence of 1 mM ATP and in the absence or presence of the indicated amounts (in micrograms/milliliter) of moxifloxacin or pipemidic acid. Reactions were stopped, and the DNA was examined by electrophoresis in 1% agarose. R and S denote relaxed and supercoiled DNA, respectively.

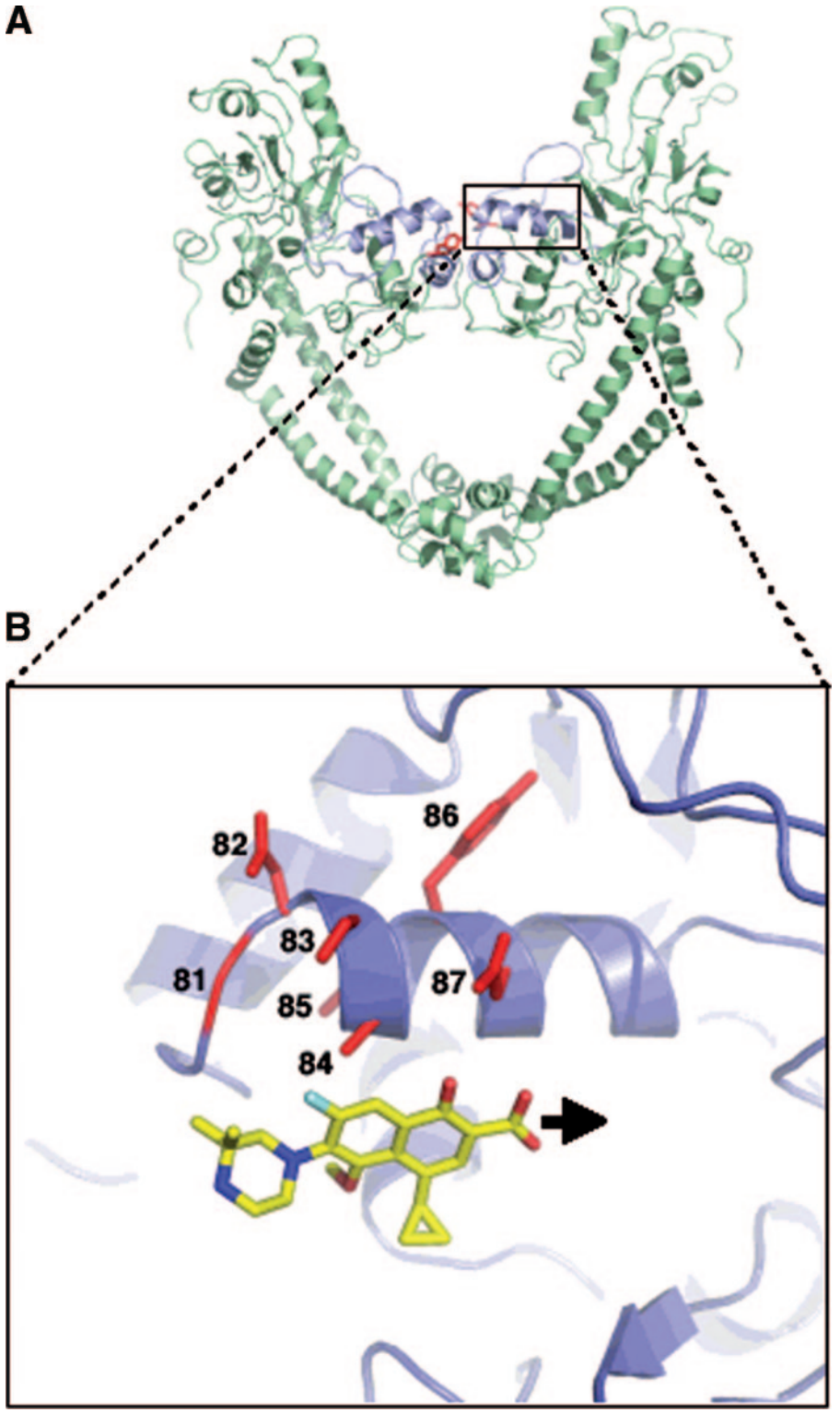

FIG. 2. (A) Ribbon representation of the 59-kDa N terminal of GyrA adapted from the crystal structure of the breakage-reunion domain of the GyrA protein of *E. coli* (16). The QRDR (quinolone resistance-determining region) is in blue, and the active site (Tyr^{122}) is in red. (B) Close-up of the region outlined by the broken box highlighting the schematic representation of the fluoroquinolone positioning in the GyrA α4 helix domain. The fluoroquinolone used for the model is gatifloxacin. The black arrow shows the displacement of the fluoroquinolone positioning in the *M. tuberculosis* GyrA α4 helix domain compared to the one proposed for *E. coli* as suggested by our study.

quinolone ring substituent, while the remaining core of the quinolone interacts with amino acids 83 and 87, the positions strongly involved in quinolone resistance (16). The same observation has been made by Sindelar et al. (21), who suggested that for *M. smegmatis*, a rapidly growing mycobacteria, the R7 ring binds to gyrase near amino acid 81. It is conceivable that modifications of gyrase such as those observed in *M. tuberculosis* WT (A83) and the mutant at position 81 (A- or C-81) could create a steric hindrance, leading to a displacement of the binding of quinolones compared to that of *E. coli* as shown in Fig. 2. Consequently, the binding of molecules with a bulky group at position R7 decreases, while no change in binding would occur with molecules like nalidixic acid and flumequine, which have a linear substituent at that position. The results obtained for the enzymes carrying mutations at position 88 bring new insights into the role of the positioning of quinolones into the quinolone binding pocket of the *M. tuberculosis* DNA gyrase.

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